NMR spectroscopy for Structural Biology
Prof. Ashutosh Kumar and Prof. Ramkrishna Hosur
Department of Chemistry
Indian Institute of Technology - Bombay

Lecture: 40
NMR Analysis of Protein Associations

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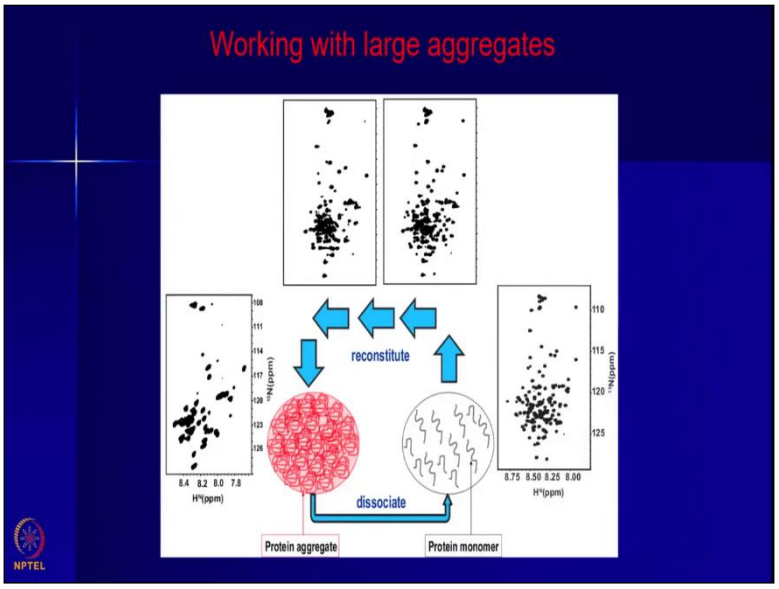

So, we continue with the discussion of aggregates, how to use NMR to understand the process of self-association. Here you have the example of the same protein, GED what was earlier and here we have an assembled state, so, an aggregated state here; and our challenge is to identify this. Each of these molecules has a certain structure and they have assembled to form a large aggregate.

Now what we should do? Well one can do you can dissociate this into the individual monomers, break the associated state into individual monomeric states and when you make it monomer of course you will see a beautiful NMR spectrum, because each one of them is a small molecule now. So, that will give you all the expected peaks, you will get here in this protein. Now you assign all of these using the standard methods, which you have discussed earlier. You assign the individual peaks.

Now what you do is you start reconstituting this. So, slowly change the conditions and then monitor the HSQC spectrum as you are going through the process of association. See therefore

a few of those once are indicated here, from here you go to this, then you go to this, and finally when you reach here, you come back to this situation where you have only small number of peaks.

Therefore, in this entire process, you can monitor the changes in the chemical shifts or the line widths of the individual peaks to tell you which residues are associating in the sequential manner. So, that is the strategy. So, that is how we can identify. Of course, when you induce this initially, this is all unfolded. Now when you dissociate it, they become unfolded, but when you start the reconstitution process, they will also start folding.

When it will start folding, of course, there will be small chemical shift changes. So, therefore you monitor the changes in the line widths and the chemical shifts to understand about the process of the self-association.

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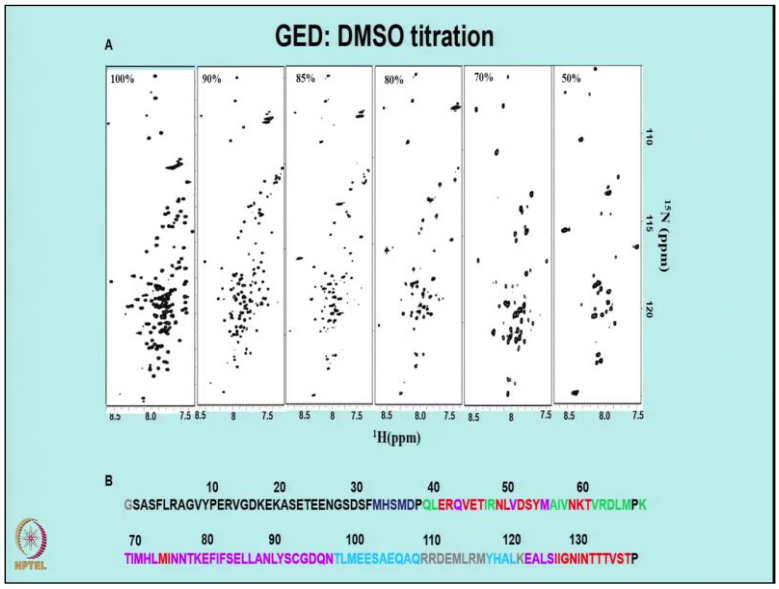

Now here is an illustration of that. So, you have the GED, GED is dissociated using DMSO the dimethyl sulfoxide. In presence of dimethyl sulfoxide, the assembly is completely dissociated. You will see all the peaks here and beautifully you can analyze all of these ones. Now you start decreasing the DMSO concentration. These are all separate samples, you will have to prepare separate samples. So, this is prepared in 100% DMSO, this is in 90% DMSO, this is 85% DMSO, and so on and so forth.

When you have 90% DMSO, you do not see as many peaks as are seen here, the few peaks have seen and many peaks have disappeared. Now you monitor those, which are the ones,

which are changed. Come to 85%, more peaks have disappeared; 80% even more have disappeared; 70% more disappeared; and in 50% almost it is like the original associated state. So, you stop it. Beyond that, it does not help anymore, because you already reached the completely associated state here.

Now you analyze this; what you get here, this is what is shown here. This portion of the protein is the N-terminal, which is what you actually see here. This remains flexible even in the associated state, this is what we saw in the previous example also and these remains associated. You will see these peaks, and these ones, which are colour coded here; these ones are disappearing in a sequential manner. Stepwise they actually disappear as you are decreasing the DMSO concentration.

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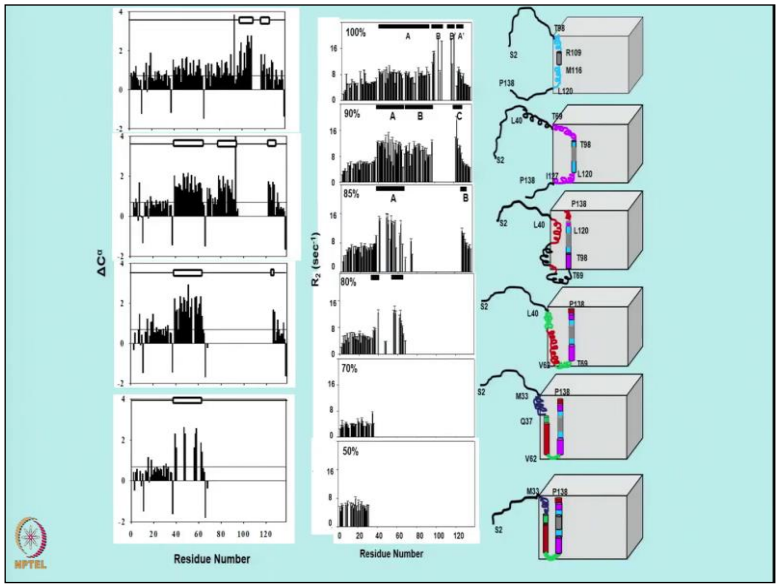

What are the changes that are happening in the protein with regard to the structure and with regard to the relaxation properties; that is shown here. So, you see here, this is the secondary structural propensities measured using the ΔCα. So, with the ΔCα, this is the secondary shift what we are measuring here and here you see that only a few residues, they are mostly down here and here it is everything in the same direction.

A few of those are quite above and these are the once where the helical propensities are there. This is indicating helical propensities, a few stretches have a helical propensity there. Now you decrease, helical propensity increases. So, this area also gets a helical propensity, this gets the helical propensity, these peaks have disappeared, you see this helix which was present, these

peaks have disappeared here, which means they are gone into the assembly, onto the associated state.

Now this one is little bit of that is remaining. So, so many peaks which are present here they have vanished here; because these are gone into the associated state. You cannot monitor those peaks because these peaks are vanished. Now we further go down to further another value of the DMSO, so you see these many peaks have disappeared. All of these are gone into the associated state and this big helix is still remaining.

This helix is still remaining and the N-terminal anyway is a free thing to do. So, this will remain till the end. So, when you go further down, these are four standard typical points are shown here. So, even from here from, this helix also so, many peaks have disappeared here. So, small number of segments are seen in this area some peaks are there but many peaks have disappeared and this is the result of the association process.

Now what happens with respect to the line widths and the relaxation properties? The relaxation properties are indicated here, so, 100%, 90%, 85%, 80, 70, and 50% there. So, the 50% again the N-terminal peaks are all seen and same here and now as you go come down from here. So, this is the area, which has on the basis of the relaxation properties the R_2 values. R_2 indicates as I said the transverse relaxation rate.

So, when there is the associated-dissociated exchange going on and that exchange produces the line broadening and the peaks will disappear and that is what is happening. So, therefore step wise it indicates which peaks are disappearing, which peaks are in the in the exchange process, and therefore that indicates the place where the association is going on.

So, as you come down see the C-terminal, this particular portion is the one which is very vulnerable and these ones are interacting and then of course you see these peaks have disappeared here. You come down to 85%. So, many peaks have disappeared because these ones have participated in the association process. Come down further, so all these have gone, even this has gone, the C-terminal has gone, some residues from here also have gone.

This way these are also gone into the association process and then by the time you reach 70% you already have vanished all these peaks which are here from here to here. So, almost from

residue number 25 till the end, all the peaks have disappeared and 50% it is the same. Now on the right side, it indicates where the helical propensities are there and how the line width changes are happening.

See this one is the N-terminal, which is present here this has the flexible portion. This is always seen here in the unfolded state and then of course, it gets a little bit of a helical propensity, when you come down in this N-terminal also. And then some helical propensities are seen for these residues, which are in the interior of the box. This box is actually representing the associated state.

So, the residue numbers are given here. So, which residues are going into the associated state and form helical propensities which are remaining flexible without a structure. So, as you go down further and further, more helices are formed, and more helices are getting into the interior of the of the aggregate and they are all disappearing. Notice, when you looked at the CD spectrum of the entire aggregate it was mostly helical.

Therefore, eventually the protein goes into the helical state. So, here all of this is helical, everything is going into the interior of this aggregate and everything is has disappeared. So, this is how the process of self-association happens and this was extremely difficult to do it and this was possible only because of the pulse sequences which are described to you earlier. These were the HNN-HNCN pulse sequences, which are able to study disorder proteins, flexible proteins.

And because that one was able to identify the individual residues in all of these individual steps.

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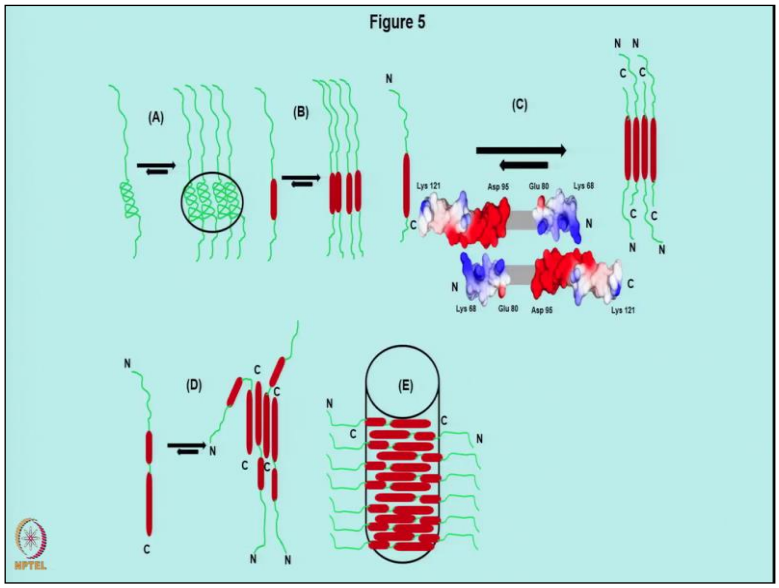

Now let us look at what is happening from the structural point of view. So, the same thing is indicated here. Now you look at this, this is the N-terminal, this has certain helical propensity here. So, you go further to the next step, these helices start aggregating, transiently they are aggregating, then of course the proper structure is formed, the stable structure is formed, the helix is formed and these helices start aggregating here, this can form a helical aggregate in this process. Now one can analyze a little bit more, what sort of an aggregate is formed or is it in this way or they are oriented in opposite directions.

Here at this point we are not able to say that, this N-terminal and the C-terminal, they are all going parallel, is that the way it is aggregating or it is any other way. So, now let us look at that. If you look at these amino acid residues which are present in these regions. Now you plot here the electrostatic charges on these individual residues. The N-terminal has a particular charge and the C-terminal has a red charge here.

These are positive-negative charges as you can see and if you lay this like this, is a complementarity comes in here, it is because of this, this association is happening the electrostatic interactions here. Positive-negative charges are coming close and that is what is causing the aggregation; which means these two chains are not going in the same direction but they are going in the opposite direction and that is what is shown here.

See the aspartate is a negatively charged residue, glutamate is the negatively charged residue, what is present here at N-terminal, this is the lysine, is it a positively charged residue. The positively charged residue is coming close to the negatively charged residue and therefore this

is the complementarity of the charges, which are there. Same thing is happening here the N-terminal lysin68, you put them in opposite orientations, this portion is this, and this portion is this, put them in opposite orientations.

This leads to the mechanism of the association process and that is what is shown here. So, you draw adjacent chains in opposite directions N-terminal to the C-terminal, N-terminal to the C-terminal, and N-terminal to the C-terminal, and things like that. Notice the extension present at the C-terminal is quite small compared to the extension present at the N-terminal. So, you see this here, and then you further go down, and then see further helix is formed, this is one particular helix.

Now you form another helix as you go next step. So, you'll have to put them in opposite directions N-terminal to the C-terminal, the entire C-terminal is covered here, entire C-terminal has formed the helical structure. This is a stepwise process as I shown you earlier and this also is a helix structure and a large N-terminal segment is flexible.

So, you draw that here. So, at the N-terminal a small helix, a long helix at the C-terminal, once again from here, put in the opposite direction; N-terminal short helix and the long helix, and these are complementary to each other; and therefore they will form an association state. Now you draw that picture in this manner to show the association which is happening. You lay them one over the other, see there is nothing almost at the C-terminal. C-terminal is completely in the helical form, the N-terminal is hanging.

So, when you do this, you get the cylinder. You lay them one above the other, you get a sort of a cylinder, which means it is like a rope. You see now the GED is forming a rope and you have these flexible N-terminals on either side. So, these are like the arms of this assembly of the helical associate. How is it useful?

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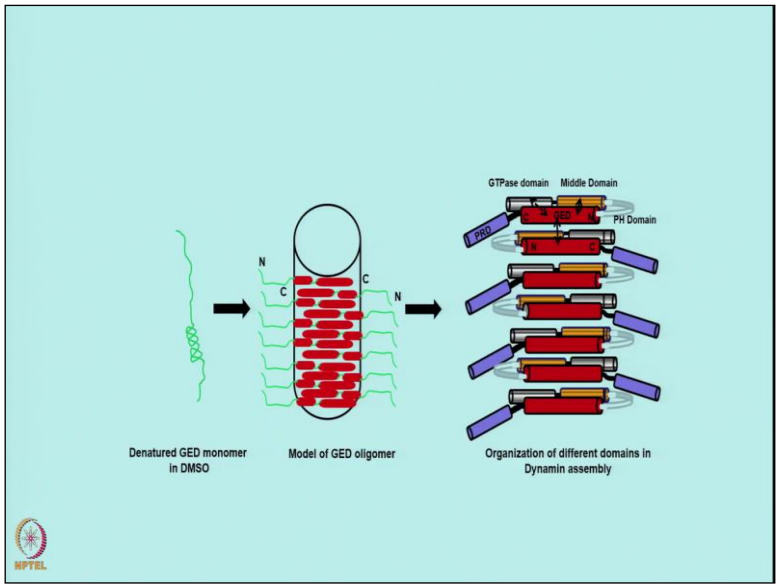

This is useful because this GED oligomer which forms a rope, which binds to the membrane at the budding vesicle. In the budding vesicle at the neck of this, this is the lipid membranes. The lipid membranes have the negative charges and we have here residues which have positive charges and they will bind to the phosphate groups of the negative charges of the lipid membranes.

Therefore, this will associate in the form of a neck and wrap around the neck of the vesicle. So, that is how this entire rope is able to bind at the neck in the tight manner. Now from these of course, we can incorporate these in entire dynamin and then you will have the organization of different domains in the dynamin assembly and the red ones are the GEDs and this is the assembly.

And other domains, you can put them aside and then you can form a rope with various domains. This comes from the certain other biochemical evidences as well. Put that together here and say you generate a model of the organization of dynamin in this form of a rope.

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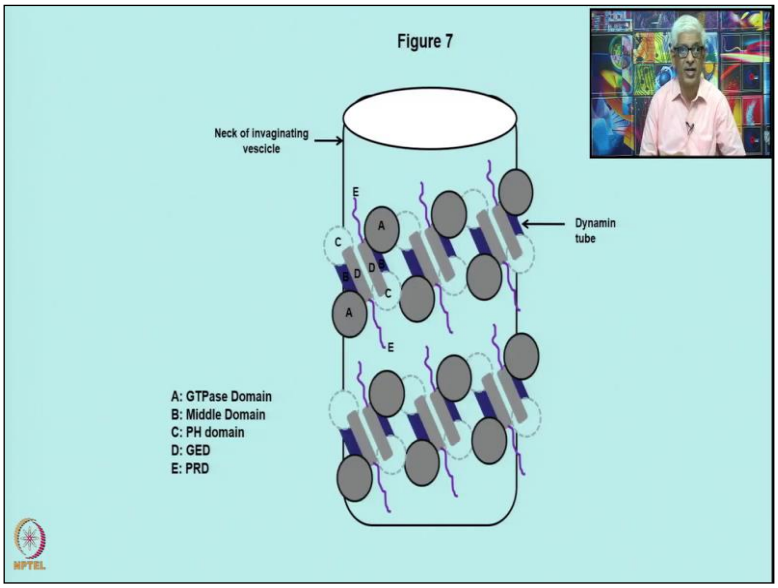

And that is what is shown here. So, this is the neck of the invaginating vesicle, this is the membrane surface here, and you draw this like this. The previous model, which I showed you here, you have the GTPase domain, middle domain, pH domain, GED and this. Here this is the GED in all of this, this is associating into this, D is the GED. A is GTPase domain and C is the pH domain.

These are from the two different molecules right, opposite sides. Therefore, this will wrap around this vesicle here, this is the dynamin tube. Therefore this is the kind of a dynamic tube; which wraps around the neck of the invaginating vesicle. That is how the process of endocytosis comes. Of course, for the biological process to happen for the suppression of this thing, for the GTP hydrolysis energy is required. GTP hydrolysis works to generate, the separate out the budding vesicle. So, that is how we study association process.

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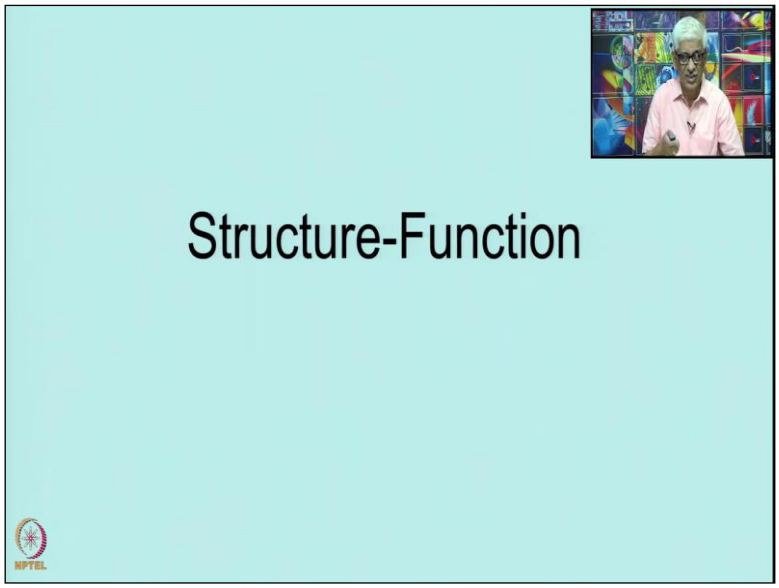

Now I will show you one example how the dynamics and the structure are important for the biological function. I already showed you in the case of HIV protease. I will show you one more example here and this is with regard to this particular protein called dynein – light chain protein.

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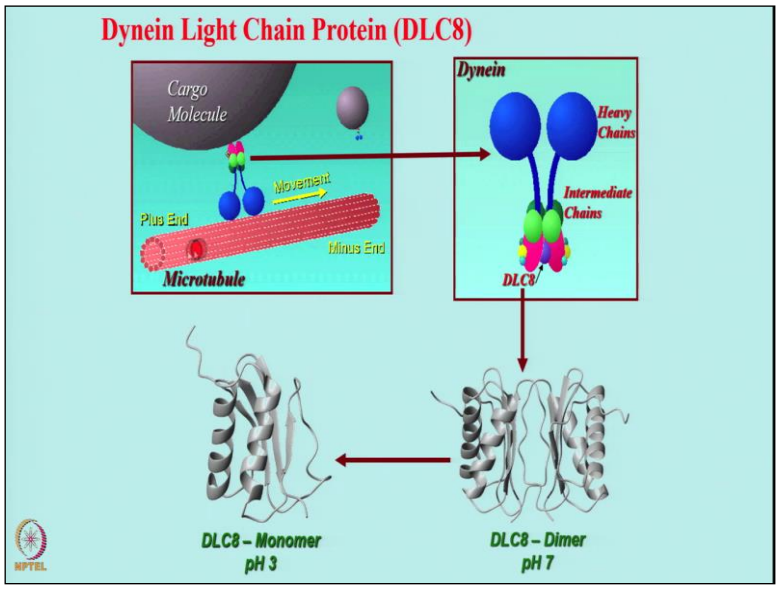

So, this is a trafficking protein, it carries cargo from one side of the cell to other side of the cell. It works on the microtubule here, this is the cargo molecule, and this is the small dynein light chain. The particular portion of this protein here, is actually binding to the cargo and carries it along on the microtubule. And the structure of this one is shown little bit more detail here. So, there is this small molecule here DLC8, this is approximately about the 80, 90 residues here.

The structure of this protein is shown here. This is this fellow, which actually binds to the cargo in the dimer form. This actually remains as a dimer at page 7, at pH3 it is monomer. Of course, at pH3, it is not functional. Because at biological pH, it is a dimer and it is the dimer, which is responsible for binding to the cargo here and that is how it carries it forward. The monomer is not able to bind, what does that mean? If you change the pH, of course you cause a transition in the structure of the molecule, and it can affect your efficacy of binding the cargo and this is biologically important. I will illustrate this to you, how NMR was helpful in understanding this.

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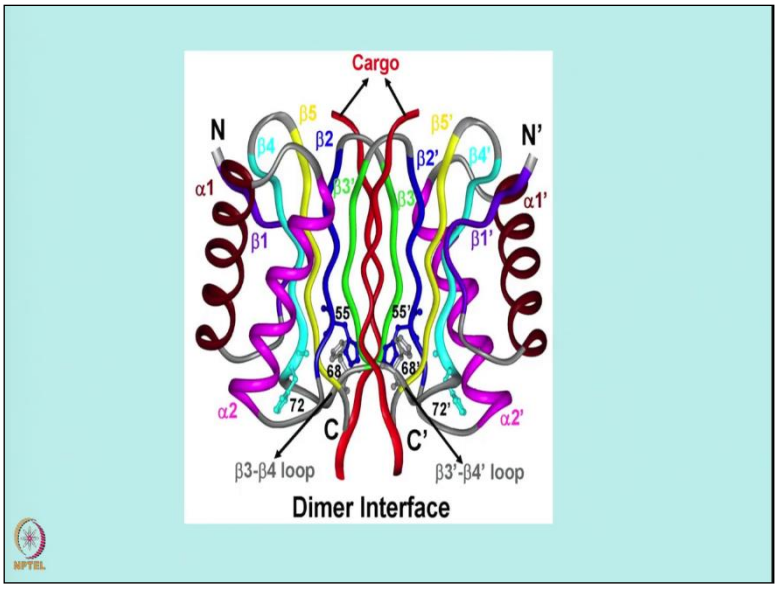

This was actually studied using a particular cargo here, with a small peptide here, somebody had this peptide here, as a cargo and you have a dimer here. So, what does the protein consist of? It consists of helices here, there is a helix, helix and then of course, you have a β-structure there. So, largely it is helical protein and you have the loops and the β-sheets going on.

So, at this point there are the β-structures and this is the dimer interface. This is the β3-β4 loop, at the dimer interface the cargo is bound. The cargo is held together at the dimer interface by this kind of interactions there. Therefore, any perturbation that happens at the dimer interface will affect the binding efficacy. Any perturbation that happens in the dynamics of the dimer, it will also affect the binding efficacy.

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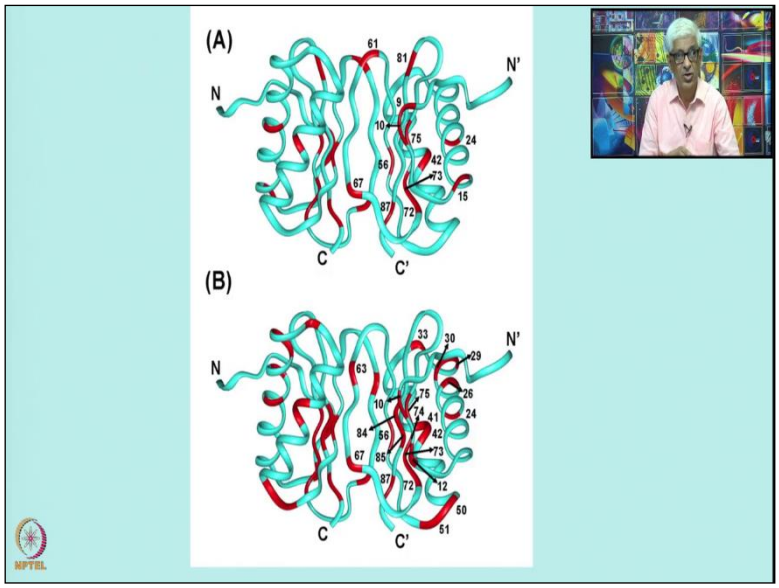

So, let us see what happens. Now we look at the dynamics of this protein, this can be studied by relaxation data by the T1-T2-NOE and all of those ones, which will be covered also separately in Professor Ashutosh's lectures. And so, here you see the red ones are the areas where there is a dynamism. This is at one particular pH, this is at another pH, this is at pH 7, this is at pH 6, so at slightly lower pH 6 or 6.5.

So, there is a change in the dynamics in the protein as we change the pH of the solution and this has important implications. This is what we will see.

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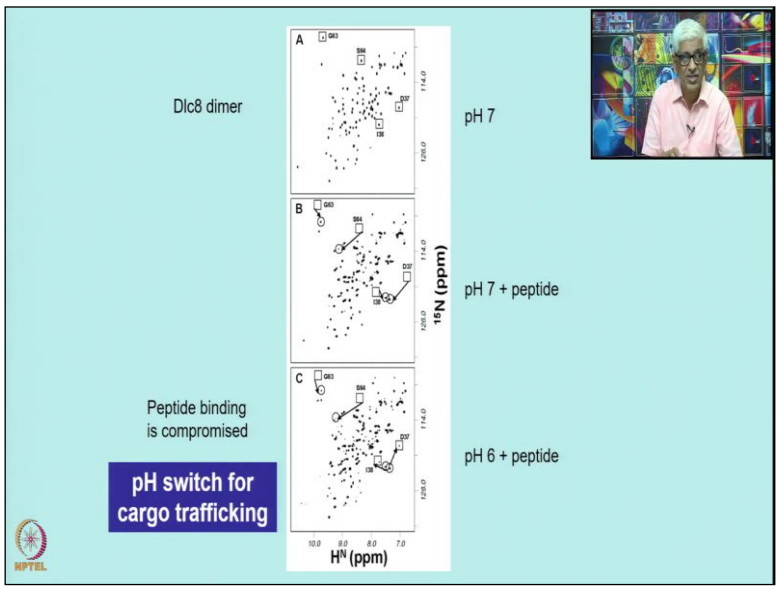

So, pH 7, this is the DLC8 dimer and this is a very beautiful spectrum as you can see. All the peaks are very well resolved and you want to focus our attention on these particular peaks. I am showing only the few peaks, but things can happen any every other place as well. Now this

is a particular four peaks, you look at it, this is G, this is serine, this is aspartate, and this is isoleucine.

So, this at pH 7, this is a very well resolved spectrum. Now if you add the peptide which is the cargo, what happens, there is a kind of a peak movement, because the peptide is binding. When the peptide is binding, of course, there is a structural change and the chemical shift changes are happening. So, this peak has moved here, this peak has moved here, and similarly this peak has moved here, this peak has moved here.

I am only showing you the four peaks, there are changes in other places as well. But just to illustrate, these four peaks are picked up. Now you do the same experiment at pH 6, you change the pH of the solution, you bring down to the pH 6, what happens see, some of these intensities come back here. So, intensity has come back here, and some intensity has come back here too. What is the meaning? It means that the binding efficacy of the peptide has reduced.

So, certain amount of free protein is produced, earlier everything was bound and once you change the pH, the binding efficacy has reduced. Therefore this peak has come back here. So, this has an important implication for the biological function as to how this protein can carry the cargo. So, this is the pH switch for cargo trafficking, if we change the pH slightly, then the binding efficacy will change.

So, why is it important, because cargo trafficking meaning what? So, the protein has to bind the cargo at some place and release it another place. What could be the mechanism for that? We are saying here that if it has to be released, a small change in the pH can do this, but how can the pH change happen, pH can happen due to some signalling. The signals can come from outside to the system as a result of which there can be a change in the dynamics.

And this is the areas where there is dynamics changes. When there is a change in the dynamics, of course, the binding efficacy will change. Why does the binding efficacy change, when you change the pH, because the protein becomes more dynamic. So, when it is so, then it is not able to bind it in the same way as it was doing when it was at pH 7.

So, therefore here the dynamics and the structure are important for bringing out a biological function. Therefore we say, this is the pH switch for cargo trafficking. There can be other

signals also; various other signals are also possible, that some other interactor will come and then cause release and things like that. But even without that, even this small change with respect to the pH, there can be acidity and things like, that various sort of things can happen and that can cause a change in the condition from one part of the cell to other part of the cell and then you will have the trafficking possible.

So, I think I have come to a close here. So, with all of this so we complete this particular portion of applications of NMR to different aspects of biological function. We described the various aspects of structure determination. We described the protein folding pathways and we described the methods earlier and from the protein folding pathways we looked at the association with the large assemblies.

And the large assemblies how to investigate using different techniques and the different techniques are of course to be used carefully at a particular magnetic fields not to lose the intensities and then the association process can be investigated by using the special pulse sequences which we described. These are the three dimensional HNN-HNCN based experiments. There are quite variations of those, which are quite useful in addressing different kinds of protein systems.

And then we also looked at how one can use this to study the folding pathways. We took two examples one we took out the SUMO protein, then we looked at the HIV protease and demonstrated that cooperativity is an important phenomena and which can be understood using NMR. How cooperativity happens; this is the process, which happens in the protein folding process and this can be analyzed and understood; which portions are cooperating in bringing about the final native state.

And then we looked at how biological structure protein structure and dynamics is responsible for the biological function. I think with that we will stop and we sort of end the course here.